Biotechnol Lett (2013) 35:407–412
DOI 10.1007/s10529-012-1095-0

ORIGINAL RESEARCH PAPER

BlpC-regulated bacteriocin production in *Streptococcus thermophilus*

John A. Renye Jr. · George A. Somkuti

Received: 26 September 2012 / Accepted: 9 November 2012 / Published online: 27 November 2012

Abstract *Streptococcus thermophilus* B59671 produces a bacteriocin with anti-pediococcal activity, but genes required for its production are not characterized. Genome sequencing of *S. thermophilus* has identified a genetic locus encoding a quorum sensing (QS) system that regulates production of class II bacteriocins. However, in strains possessing this gene cluster, production of bacteriocin like peptides (Blp) was only observed when excess pheromone was provided. PCR analysis revealed this strain possessed *blpC,* which encodes the 30-mer QS pheromone. To investigate if BlpC regulates bacteriocin production in *S. thermophilus* B59671, an integrative vector was used to replace *blpC* with a gene encoding for kanamycin resistance and the resulting mutant did not inhibit the growth of *Pediococcus acidilactici*. Constitutive expression of *blpC* from a shuttle vector restored the bacteriocin production, confirming the *blp* gene cluster is essential for bacteriocin activity in *S. thermophilus* B59671.

Keywords Bacteriocin · Quorum sensing · *Streptococcus thermophilus* · Thermophilin 110

Introduction

Lactic acid bacteria (LAB) which are used in the production of fermented foods have been designated as GRAS (generally regarded as safe) organisms and their bacteriocins, which are small, ribosomally encoded, antimicrobial peptides, have garnered attention as potential food-grade biopreservatives (Mills et al. 2011). However, nisin, a lantibiotic naturally produced by select strains of *Lactococcus lactis* subsp. *lactis,* remains the only bacteriocin approved for use as a food ingredient by the US Food and Drug Administration (Federal Register 1988).

Several strains of *Streptococcus thermophilus*, a thermophilic LAB used in the production of yogurt and cheeses, produce bacteriocins with some displaying broad spectrum activity against food-borne pathogens and spoilage bacteria (Ward and Somkuti 1995; Villani et al. 1995; Marciset et al. 1997; Ivanova et al. 1998; Mathot et al. 2003; Gilbreth and Somkuti 2005; Kabuki et al. 2009). Of these bacteriocin-producing strains, the genetic elements required for expression

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-012-1095-0) contains supplementary material, which is available to authorized users.

J. A. Renye Jr. (✉) · G. A. Somkuti
United States Department of Agriculture, Eastern Regional Research Center, Agricultural Research Service, 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA
e-mail: john.renye@ars.usda.gov

have only been reported for thermophilin 13 (Marciset et al. 1997) and the lantiobiotic, thermophilin 1277 (Kabuki et al. 2009). Through the use of comparative genomics, a gene cluster resembling part of the bacteriocin-like peptide (*blp*) locus of *Streptococcus pneumoniae* (de Saizieu et al. 2000) was identified in three non-producing strains of *S. thermophilus*: LMD-9, CNRZ1066 and LMG18311 (Hols et al. 2005). Genes encoding for potential bacteriocins were identified by the presence of a two glycine (Gly) leader peptide required for secretion; and the components of a regulatory quorum sensing (QS) system were encoded on two operons. One operon consisted of three genes encoding an ABC-transporter (*blp*A), a transport accessory protein (*blp*B), and a QS pheromone (*blp*C); the second operon contained two genes encoding a histidine kinase (*blp*H) and response regulator (*blp*R). For strains LMG18311 and CNRZ1066, the lack of bacteriocin production was based on the presence of a truncated BlpB which prevented the processing and secretion of the QS pheromone (Hols et al. 2005; Fontaine et al. 2007). In *S. thermophilus* LMD9, all components of the QS system were intact, and overexpression of *blpC* from a multicopy plasmid or supplementation of the growth medium with the BlpC pheromone, elicited bacteriocin production. Further analysis of the *blp* gene cluster in LMD9 revealed that the transcript for *blpABC* was unstable, thus the pheromone was unable to accumulate and turn on bacteriocin production under standard growth conditions (Fontaine et al. 2007).

This study was carried out to investigate if the BlpC-mediated QS system was required for the production of a broad spectrum bacteriocin constitutively expressed by *S. thermophilus* B59671. The initial characterization of this bacteriocin showed a dramatic increase bacteriocin production at mid- to late-growth phase when the culture reached an OD_{600} (Gilbreth and Somkuti 2005). This may represent a population density where the accumulation of QS pheromone has surpassed a threshold level, resulting in the production of bacteriocin. To test this hypothesis, an integrative vector was constructed for removing *blpC* from the *S. thermophilus* B59671 genome without disrupting the other components of the *blp* gene cluster. The results of this study will provide novel information on whether BlpC has a regulatory role in *S. thermophilus* strains designated as bacteriocin producers.

Materials and methods

Bacterial strains and plasmids

Streptococcus thermophilus B59671 (NRRL, Agricultural Research Service Culture Collection, NCAUR-USDA, Peoria, IL, USA) and *Enterococcus durans* 41D (laboratory collection) were grown in tryptone/yeast extract/lactose (TYL) broth, pH 6.5 at specified temperatures, and *Pediococcus acidilactici* strain F (gift from B. Ray, University of Wyoming) was grown in deMan, Rogosa and Sharpe medium (MRS) at 37 °C. *Escherichia coli* DH5α was grown in brain heart infusion (BHI) medium at 32 °C. When appropriate, TYL was supplemented with 15 μg erythromycin/ml or 150 μg kanamycin/ml; and BHI was supplemented with 150 μg erythromycin/ml. The integrative vector pKSI was a gift from K. Shatalin (New York University School of Medicine), and construction of the shuttle vector pMEU5a was described previously (Solaiman and Somkuti 1993).

DNA cloning procedures

Restriction endonucleases, T4 DNA ligase and Taq polymerase were purchased from New England BioLabs (Beverly, MA, USA) and PCR primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). PCR amplification of DNA fragments was performed using the oligonucleotide primers listed in Supplementary Table 1 and the following protocol: 5 min at 95 °C; followed by 35 cycles of 95 °C for 30 s, 45–50 °C for 30 s and 74 °C for 1 min/kb DNA; with a final extension at 74 °C for 5 min. PCR products were visualized on a 1 % agarose gel in TAE (0.04 M Tris/0.02 M acetic acid/0.001 M EDTA, pH 8.0) and purified using the QIAquick PCR purification kit (Qiagen Inc, Valencia, CA, USA). Nucleic acid sequencing of amplified DNA fragments was performed using an ABI PRISM 3730 (Perkin-Elmer, Wellesley, MA, USA) DNA analyzer with ABI PRISM Big Dye terminator cycle sequencing reagent, and further analyzed using Sequencher 4.9 (Genes Codes Corp, Ann Arbor, MI, USA).

The overlapping PCR protocol used to fuse the DurA leader peptide with the 30-mer QS induction peptide of BlpC was carried out in two steps. An initial PCR reaction was used to fuse the *durA* DNA fragment amplified with the Sec 1 and 2 primer pair,

and the *blpC* fragment amplified with the BlpC 2 and 3 primer pair. The *blpC* DNA fragment contained 24 nucleotides homologous to the 3′-end of the durA PCR product which allowed for their fusion using the following PCR conditions: 95 °C 5 min; followed by 16 cycles of 95 °C for 30 s, 50 °C for 30 s and 74 °C for 45 s. After these initial 16 cycles the PCR reaction was paused and the Sec 1 and BlpC 2 primers were added to amplify the *durA/blpC* fusion product using the following conditions: 35 cycles of 95 °C for 30 s, 48 °C for 30 s and 74 °C for 45 s; with a final extension at 74 °C for 5 min. The final product was analyzed by nucleic acid sequencing to confirm proper fusion of the DNA fragments.

Plasmids were isolated from *E. coli* DH5α using the Qiagen Midi kit or by alkaline lysis followed by CsCl/ethidium bromide ultracentrifugation (Stougaard and Molin 1981). Plasmid transformation was performed by heat-shock (Sambrook et al. 1989) using freshly prepared *E. coli* competent cells; or by electrotransformation into *S. thermophilus* (Somkuti and Steinberg 1988).

Bacteriocin assays

Bacteriocin production was measured using an agar diffusion method described previously (Gilbreth and Somkuti 2005). Briefly, *S. thermophilus* cultures were grown overnight in TYL medium at 37 °C, after which the cell-free supernatants were collected. Cell-free supernatants were loaded into precast wells (45 µl) in MRS agar seeded with *P. acidilactici* F (0.3 % v/v). Plates were allowed to equilibrate at 4 °C for 3 h, and then incubated at 37 °C and examined for zones of inhibition. Agar diffusion assays were repeated a minimum of three times.

Results and discussion

Construction of pSTKOC

Initially primers were designed based on the reported *blpC* sequence from *S. thermophilus* LMD9 (Makarova et al. 2006) to check if the gene was present in the *S. thermophilus* B59671 genome. The primer pair, BlpC 1 and 2, successfully amplified a 0.19 kb DNA fragment from *S. thermophilus* B59671 (data not shown), and subsequent nucleic acid sequence analysis confirmed that it encoded a 53 amino acid peptide identical to the BlpC peptide reported for LMD9 (Fontaine et al. 2007). The presence of *blpC* was also reported in the first two *S. thermophilus* genomes completely sequenced, CNRZ1066 and LMG18311 (Bolotin et al. 2004), and was identified in the recently sequence strains NDO3 (Sun et al. 2011), MN-ZLW-002 (Kang et al. 2012) and JIM8232 (Accession number: NC 017581). These data show that *blpC* is highly conserved within *S. thermophilus* strains, yet its role as a QS pheromone, regulating the production of a bacteriocin, has only been demonstrated in LMD9 when the concentration of BlpC was artificially increased. Overexpression of *blpC* from a multi copy plasmid or the addition of a synthetic 30-mer fragment BlpC was shown to induce bacteriocin expression in LMD9 (Fontaine et al. 2007), but a regulatory role for BlpC has not been shown for *S. thermophilus* strains designated as natural bacteriocin producers.

S. thermophilus B59671 produces a broad spectrum bacteriocin called thermophilin 110, but the genes encoding this bacteriocin and proteins regulating its expression have not been identified. The kinetics of thermophilin 110 production, which showed optimal expression at a high cell density (Gilbreth and Somkuti 2005), suggest that bacteriocin production may be regulated by a quorum sensing system. To investigate if BlpC played a regulatory role in the production of thermophilin 110, the integrative vector pSTKOC was constructed to remove *blpC* from the *S. thermophilus* B59671 chromosome without disrupting other components of the blp gene cluster (Fig. 1). PCR primers were designed to amplify the 3′-end of *blpB* and *blpH* from this strain, since it was reported that these genes are located immediately upstream and downstream of *blpC,* respectively, in the characterized blp gene clusters of LMD9, CNRZ1066 and LMG18311 (Hols et al. 2005). The BlpH 1 and 2 primer pair amplified a 719 bp DNA fragment from B59671 that included the 3′ end of *blpH,* and contained *Kpn*I and *Xho*I recognition sequences. Following digestion with these restriction endonucleases the *blpH* DNA fragment was inserted within the multiple cloning site (MCS) of pKS1, located downstream of the kanamycin resistance gene (Shatalin and Neyfakh 2005). Next, the BlpB 1 and 2 primer pair were used to amplify a 726 bp DNA fragment of *blpB* from B59671, which contained recognitions sequences for *Spe*1 and *Pst*I,

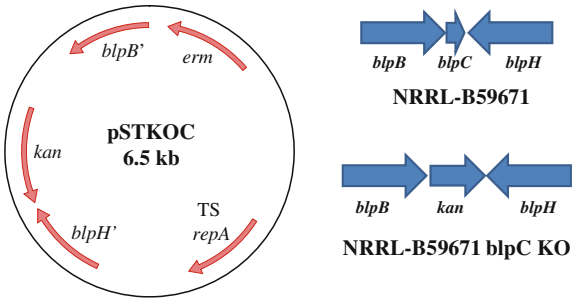

Fig. 1 Plasmid map of integrative vector pSTKOC, and an illustration of the *S. thermophilus* B59671 *blp* locus following replacement of *blpC* with a kanamycin resistance gene *(kan)*. The *blpB* and *blpH* include the 3′ end of each gene; TS *RepA* is the temperature sensitive origin of replication; and *erm* encodes for erythromycin resistance. The illustration of the chromosomal blp locus shows that kan was inserted in the same orientation as *blpC* in the parent strain, and that both *blpB* and *blpH* were reconstituted after insertion of *kan*

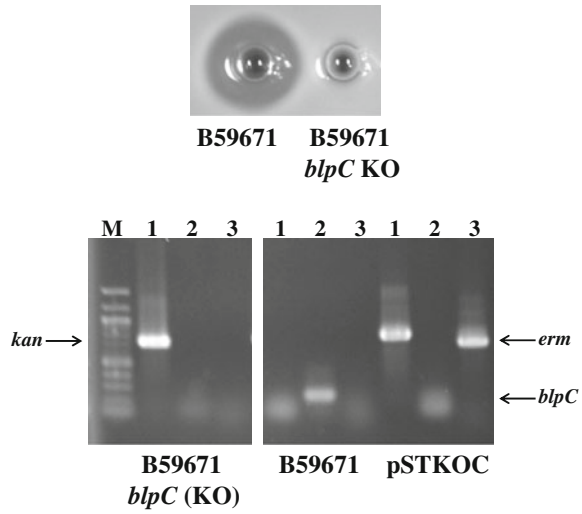

Fig. 2 Effect of removing blpC from the S. thermophilus B59671 chromosome. Bacteriocin activity (top) was measured by well-diffusion assay with using *Pediococcus acidilactici* F as the target bacterium. PCR confirmation of the double homologous recombinant event for replacing *blpC* with kan in the *blp* locus. *Left* to *Right*: *S. thermophilus* B59671 mutant (*blpC*$^{-}$); *S. thermophilus* B59671 parent strain; pSTKOC. Amplification products: *Lane 1*: kan (kanamycin resistance); *Lane 2*: *blpC*; *Lane 3*: *erm* (erythromycin resistance)

allowing for its insertion within pKS1-*blpH* at the corresponding sites located in the MCS upstream of the kanamycin resistance gene. In the resulting plasmid, pSTKOC, the *blpH* and *blpB* gene fragments were positioned in opposite orientation, similar to how they were reported to reside on the chromosomes of LMD9, CNRZ1066 and LMG18311 (Hols et al. 2005). This was essential to ensure that the chromosomal copies of *blpB* and *blpH* remained intact after the two homologous recombination events (Fig. 1). Proper construction of pSTKOC was confirmed by digeston with *Kpn*I, which resulted in the presence of two DNA fragments of 2.3 and 4.1 kb (data not shown).

Inactivation of *blpC* in *S. thermophilus* B59671

S. thermophilus B59671 was electrotransformed with pSTKOC, and transformants were selected for on TYL agar containing erythromycin at 30 °C, a permissive temperature for the replication of pKS1-derrived plasmids in *S. thermophilus* (Renye and Somkuti 2012). Transformants were screened by PCR with the Kan Fwd and Kan Rev primer pair, which resulted in the amplification of a 715 bp DNA fragment confirming the presence of pSTKOC. The transformants were then subcultured at 42 °C in TYL broth with Em to allow for a single crossover event at either *blpB* or *blpH*. Clones with the integration occurring within *blpB* were identified by PCR using the BlpC 2 and Erm Rev primers, which amplified a 1.7 kb DNA fragment. Integration within *blpH* was confirmed in only one clone as a 2.3 kb DNA fragment was amplified with the BlpC 1 and TS Rep 1primers. Clones in which a single homologous recombination event occurred were grown at 30 °C (five passages) in the absence of antibiotic to allow for the second recombination event to occur. The cultures were then transferred to TYL containing kanamycin and grown at 37 °C; cultures that grew were transferred to TYL containing Em and incubated at 37 °C. Cultures that grew in the presence of kanamycin and not in EM were chosen to be screened for bacteriocin production with *P. acidilactici* F as the target bacterium. Of the cultures screened, six had lost their anti-pediococcal activity as evidenced by the lack of inhibition zones on a well-diffusion assay (Fig. 2). PCR analysis was used to further confirm the removal of *blpC* by a double homologous recombination event within the *S. thermophilus* B59671 chromosome. In the cultures lacking anti-pediococcal activity the 715 bp DNA fragment, corresponding to the kanamycin-resistance gene, was amplified but *blpC* and the erythromycin resistance gene were not, confirming their removal from the chromosome (Fig. 2). In comparison, only *blpC* could

be amplified by PCR in the parent strain *S. thermophilus* B59671.

Constitutive expression of BlpC in *S. thermophilus* B59671 restores bacteriocin activity

To ensure that the loss of bacteriocin activity was due to the removal of *blpC* from the *S. thermophilus* B59671 chromosome, a shuttle vector was constructed to constitutively express BlpC in this mutant. Overlapping PCR was used to create a fusion gene encoding the 30-mer QS peptide of BlpC (Fontaine et al. 2007) fused with the 28-mer leader peptide from durancin GL (*sec*) to allow for secretion of the QS peptide via the *S. thermophilus* general secretory pathway (Du et al. 2012). Normally the QS peptide is processed and secreted by BlpA and BlpB, however, the use of an alternate leader peptide separated the processes of QS peptide and bacteriocin production. The fusion gene was cloned into pUC18, downstream of a previously cloned *S. thermophilus* 2201 promoter which has been shown to regulate the constitutive expression of recombinant genes in ST (Somkuti and Solaiman 1997). The P2201/*sec/blpC* construct was amplified with the P2201 and BlpC 4 primer pair and subcloned into the shuttle vector pMEU5a (Solaiman and Somkuti 1993), resulting in the vector pSTblpC (Fig. 3). Proper construction of the vector was confirmed by PCR amplification and subsequent nucleic acid sequencing of the P2201-*sec-blpC* fragment (data not shown). The expression vector was introduced into the *S. thermophilus* B59671 *blpC* knock out mutant by electroporation, with transformants selected on TYL Em. Potential transformants were initially screened by PCR and the 376 bp fragment corresponding to the P2201/*sec/blpC* construct was successfully amplified in six clones. All six clones also produced bacteriocin when cell-free supernatants were used in a well-diffusion assay with *P. acidilactici* F as the target bacterium (Fig. 3). It was noted that the constitutive expression of BlpC in the mutant strain resulted in smaller inhibition zones (wells 3–8) when compared to the parent *S. thermophilus* B59671 culture (well 1). It is believed that this is due to the constitutive expression of *blpC* from pSTblpC; when normally expression of the *blpABC* operon is induced as the QS threshold has been reached, resulting in increased production of bacteriocin. This result also confirms that *blpB* and *blpH*

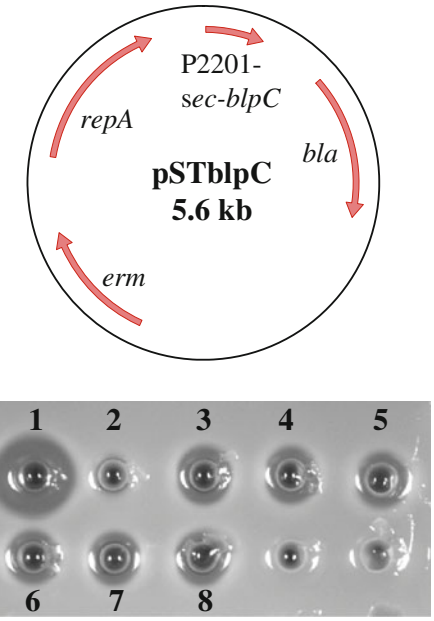

Fig. 3 Effect of constitutive *blpC* expression from pSTblpC on bacteriocin expression in knock out mutant. Plasmid map of pSTblpC (top). *bla* and *erm* encode for ampicilin and erythromycin resistance genes respectively; *repA*: gram-positive origin of replication; P2201-*sec-blpC*: fusion gene encoding DurA leader peptide fused to 30-mer BlpC, under transcriptional regulation of the *S. thermophilus* 2201 promoter. Bacteriocin expression measured by well-diffusion assay (bottom): Well 1: *S. thermophilus* B59671 parent strain; Well 2: *blpC*$^{-}$ mutant strain; Wells 3–8: six clones of *blpC*$^{-}$ strain transformed with pSTblpC

were reconstituted correctly following the double crossover event, as the mutant strain could process and secrete an active bacteriocin (BlpB-mediated) and respond to the constitutive expression of BlpC (BlpH-mediated).

To our knowledge this is the first report that showed the BlpC-mediated QS system naturally regulating the expression of a broad spectrum bacteriocin in a strain of *S. thermophilus*. Previous studies which focused on this QS system required the addition of the BlpC peptide, either by overexpression from a multi copy plasmid or as a component of the growth medium, to activate bacteriocin production. It is believed that further studies on the QS systems from B59671 and LMD9 would provide valuable information to determine the regulatory elements which allow this QS-system to function properly in *S. thermophilus*.

Acknowledgments We thank D. Steinberg, M. Toht and E. Lawrence (St. Joseph's University) for technical assistance

with plasmid and strain development, and D. Needleman for assistance with nucleic acid sequencing.

References

Bolotin A, Quinquis B, Renault P, Sorokin A et al (2004) Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol 22:1554–1558

de Saizieu A, Gardes C, Flint N, Wagner C et al (2000) Microarray-based identification of a novel *Streptococcus pneumoniae* regulon controlled by an autoinduced peptide. J Bacteriol 182:4696–4703

Du L, Somkuti GA, Renye JA Jr (2012) Molecular analysis of the bacteriocin-encoding plasmid pDGL1 from *Enterococcus durans* and genetic characterization of the durancin GL locus. Microbiology 158:1523–1532

Federal Register (1988) Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Fed Reg 54: 11247–11251

Fontaine L, Boutry C, Guedon E, Guillot A et al (2007) Quorum-sensing regulation of the production of Blp bacteriocins in *Streptococcus thermophilus*. J Bacteriol 189: 7195–7205

Gilbreth SE, Somkuti GA (2005) Thermophilin 110: a bacteriocin of *Streptococcus thermophilus* ST110. Curr Microbiol 51:175–182

Hols P, Hancy F, Fontaine L, Grossiord B et al (2005) New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. FEMS Microbiol Rev 29:435–463

Ivanova I, Miteva V, Stefanova Ts, Pantev A et al (1998) Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. Int J Food Microbiol 42:147–158

Kabuki T, Uenishi H, Seto Y, Yoshioka T, Nakajima H (2009) A unique lantibiotic, thermophilin 1277, containing a disulfide bridge and two thioether bridges. J Appl Microbiol 106:853–862

Kang X, Ling N, Sun G, Zhou Q, Zhang L, Sheng Q (2012) Complete genome sequence of *Streptococcus thermophilus* strain MN-ZLW-002. J Bacteriol 194:4428–4429

Makarova K, Slesarev A, Wolf Y, Sorokin A et al (2006) Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci 103:15611–15616

Marciset O, Jeronimus-Stratingh MC, Mollet B, Poolman B (1997) Thermophilin 13, a nontypical antilisterial poration complex bacteriocin, that functions without a receptor. J Biol Chem 272:14277–14284

Mathot AG, Beliard E, Thuault D (2003) *Streptococcus thermophilus* 580 produces a bacteriocin potentially suitable for inhibition of *Clostridium tyrobutyricum* in hard cheese. J Dairy Sci 86:3068–3074

Mills S, Stanton C, Hill C, Ross RP (2011) New developments and applications of bacteriocins and peptides in foods. Annu Rev Food Sci Technol 2:299–329

Renye JA Jr, Somkuti GA (2012) Vector-mediated chromosomal integration of the glutamate decarboxylase gene in *Streptococcus thermophilus*. Biotechnol Lett 34:549–555

Sambrook J, Maniatis T, Fritsch EF (1989) Molecular cloning a laboratory manual, 2nd edn. Cold Spring Harbor Lab Press, New York

Shatalin KY, Neyfakh AA (2005) Efficient gene inactivation in *Bacillus anthracis*. FEMS Microbiol Lett 245:315–319

Solaiman DK, Somkuti GS (1993) Shuttle vectors developed from *Streptococcus thermophilus* native plasmid. Plasmid 30:67–78

Somkuti GA, Solaiman DK (1997) ST_{P2201}, a chromosomal promoter sequence of *Streptococcus thermophilus*. Curr Microbiol 35:180–185

Somkuti GS, Steinberg DH (1988) Genetic transformation of *Streptococcus thermophilus* by electroporation. Biochimie 70:579–585

Stougaard P, Molin S (1981) Vertical dye-buoyant density gradients for rapid analysis and preparation of plasmid DNA. Anal Biochem 118:191–193

Sun Z, Chen X, Wang J, Zhao W et al (2011) Complete genome sequence of *Streptococcus thermophilus* strain ND03. J Bacteriol 193:793–794

Villani F, Pepe O, Mauriello G, Salzano G et al (1995) Antilisterial activity of thermophilin 347, a bacteriocin produced by *Streptococcus thermophilus*. Int J Food Microbiol 25:179–190

Ward DJ, Somkuti GA (1995) Characterization of a bacteriocin produced by *Streptococcus thermophilus* ST134. Appl Microbiol Biotechnol 43:330–335